

Effects of heat treatment on fluorescence properties of humic substances from sandy soil in arid land and their Hg(II) binding behaviors

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Abstract The topsoil temperature in arid areas of Xinjiang, China can be up to about 80°C in summer. This may significantly affect the chemical properties of soil humic substances. However, the effects of high temperature on characteristics of soil humic substances and their complexation with toxic metals are still poorly known. In the present study, binding of Hg(II) to unheated soil humic substances and heated soil humic substances from sandy soils was comparatively investigated using three-dimensional excitation–emission matrix (EEM) fluorescence spectroscopy. Two fluorescent peaks (peak I at $E_x/E_m = 365\text{--}370/470\text{--}474$ nm; peak II at $E_x/E_m = 270\text{--}275/468\text{--}472$ nm) identified as humic-like fluorescence were observed in the EEM spectra of humic substances. Both peaks were clearly quenched by Hg(II), indicating the strong interaction of humic-like substances with Hg(II), and showed blue shifts after heat treatment. Heat treatment caused an increase of the fraction of accessible fluorophore (f_a), binding sites number

(n) and effective quenching constants ($\log K_a$), indicating that more binding sites in humic substances could bind Hg(II) and form more stable humic substances–Hg(II) complexes after heat treatment. However, a decrease of binding constants ($\log K_b$) suggested that heat treatment would reduce the binding capacity of each binding site of humic substances to Hg(II). This study implies the transport of Hg(II) may be affected by high temperature in the arid zone due to the modification of the physicochemical properties of humic substances in soil.

Keywords Humic substances · Fluorescence quenching · EEM · Mercury · Heat treatment

Introduction

Mercury (Hg), a global toxic contaminant, has been listed as a priority pollutant by many governments and international organizations such as UNEP, WHO and FAO (Zhang et al. 2006). It is a highly mobile heavy metal, and the soil can act as a sink or source in the geochemical/biogeochemical cycle of Hg (Grimaldi et al. 2008; Feng and Qiu 2008). The fate and transport of Hg are strongly influenced by its interaction with organic ligands in the aquatic and terrestrial environments (Miller et al. 2009). The organic matters are recognized as the most important soil component controlling Hg retention behavior in soils (Yin et al. 1997; Yang et al. 2007).

Humic substances, heterogeneous mixtures of a variety of organic compounds, are ubiquitous in water, sediments and soils (Baker 2001). It has been reported that they account for 70–80% organic matters in soil (Provenzano et al. 2004). Humic substances have a strong ability to bind Hg(II) due to their high contents of oxygen-containing

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functional groups, such as carboxyl, hydroxyl, and carbonyl (Senesi 1990; Provenzano et al. 2004). Therefore, they may affect the concentration, chemical forms and bioavailability of Hg in the environment. For example, both humic and fulvic acids show an inhibitory effect on the surface Hg emission (Mauclair et al. 2008). Wang et al. (1997) found that humic substances in soil could complex strongly with Hg(II) and reduce their mobility and availability from soil to plants.

Xinjiang Uygur Autonomous Region, lying in the northwest China, is a typical arid area where topsoil is usually exposed to high temperature in summer. Topsoil temperature in parts of this area is ordinarily up to 80°C in summer days. Soil temperature is described as an important factor controlling Hg emission from soil (Song and Van Heyst 2005). Rinklebe et al. (2010) had assessed factors controlling mercury emission in a large Hg-polluted floodplain area, and found that Hg emission rates increased exponentially with soil temperatures in both laboratory and field experiments. However, the effects of high temperature on the properties of humic substances and their interaction with Hg have been poorly known.

The three-dimensional fluorescence excitation–emission matrix (EEM) spectroscopy provides qualitative and quantitative information on fluorescent functional groups of organic compounds in the form of an EEM of fluorescence intensity as a function of excitation and emission wavelength (Mobed et al. 1996; Provenzano et al. 2004; Sierra et al. 2005). EEM is a rapid and sensitive method for studying the chemical properties of fluorescent organic compounds and their interaction with heavy metals (Sierra et al. 2005; Corrado et al. 2008; Nakashima et al. 2008; Shirshova et al. 2009). Fluorescence quenching titration has been successfully used to determine the complexing capacity of fluorescent organic compounds with metals and organic pollutants (Esteves da Silva et al. 1998; Lu and Jaffe 2001; Fu et al. 2007; Zhang et al. 2010). Since humic substances are of intrinsic fluorescence (Esteves da Silva et al. 1998), EEM can be employed to investigate the chemical properties of humic substances and their binding to Hg(II).

The objectives of the present study are to examine the effects of high temperature on the fluorescence feature of humic substances from sandy soil and their binding ability to Hg(II) using EEM fluorescence spectroscopy.

Materials and methods

Sampling

The sampling site (87°55′36″E, 40°41′19″N) is located in lower reaches of Tarim River, the edge of Takla Makan

Desert, Xinjiang Uygur Autonomous Region. The topsoil (0–15 cm) samples submersed in water that were not exposed to high temperature were collected using a stainless steel spatula. More than 5-kg soil samples were collected randomly from the sampling site. The soil samples were mixed thoroughly prior to experiment. The soil pH was 7.69. The content of dissolved organic carbon (DOC) was 39.1 mg L⁻¹ and the electric conductivity (EC) was 1.12 mS cm⁻¹. All soil samples were air-dried at room temperature in the shade for 1 week and were sieved through a 0.50 mm (35 meshes) polyethylene sieve to remove large debris, stones, and pebbles. The soil samples were then heated in the oven at 80°C for 60 h.

Humic substances extraction

Humic substances were extracted from the soils in accordance with the modified method suggested by the International Humic Substances Society (Zaccone et al. 2009; Zhang et al. 2009). Briefly:

1. *Alkali-soluble compounds* The soil samples were extracted with a solution of 0.1 M NaOH and 0.1 M Na₄PO₇ at a sample to extractant ratio of 1:5. The mixtures were shaken mechanically at 200 rpm and 27°C for 8 h and then placed aside overnight.
2. *Acidation* The alkaline supernatant solution was separated from the residues by centrifugation at 8,000 rpm and 20°C for 30 min. The new alkaline supernatant solution was acidified by 3 M HCl to pH 1 with continuous stirring. Then the solution was left to stand for about 8 h to permit coagulation of the humic substances.
3. *Solidification* The coagulated humic substances were separated from the solutions by centrifugation at 12,000 rpm and 20°C for 30 min. After centrifugation, the humic substances were dried at 35°C.

Preparation of humic substances and Hg(II) solutions

Humic substances solutions were prepared by dissolving the extracted humic substances samples in 0.1 M KOH stock solution. The concentration of two humic substances solution was adjusted to the same concentration (64 mg L⁻¹, w:v) and then filtrated through 0.45-μm membrane. All solutions were stored at 4°C in the dark until use.

All chemicals used in this study were of analytical grade. The 0.05 M stock Hg(II) solution was prepared by dissolving 1.358 g of HgCl₂ in 100 ml of ultrapure water.

The fluorescence quenching titrations

The fluorescence spectra were determined on a spectrofluorometer (F-7000; Hitachi, Japan) at a $25 \pm 0.1^\circ\text{C}$ with xenon arc lamp and quartz cell ($1 \times 1 \text{ cm}$). For these measurements, the photomultiplier voltage was set at 700 V, and the corrected spectra and shutter control were set on. The EEM spectra were recorded with 1,200 nm/min and autoresponse (2 s). The scan ranges were 200–400 nm for excitation (Ex: slit width, 5 nm) and 200–600 nm for emission (Em: slit width, 10 nm) for humic substances. The spectrum of 0.1 M KOH solution was recorded under the same conditions and subtracted from the EEM spectra of the humic substances samples. The measurements were conducted in triplicate and the results were presented as the mean \pm SE

One microliter of 0.05 M Hg(II) solution was added incrementally into the humic substances solutions. After each addition, the solution was fully mixed using a

magnetic stirrer for 15 min and fluorescence spectrum was recorded. The fluorescence intensities of peaks were recorded by EEM fluorescence spectroscopy. EEM data were processed using the softwares SigmaPlot 11.0 (Systat, USA) and Origin 8.0 (Origin lab, USA).

Results and discussion

Effect of heat treatment on the fluorescence spectroscopy of humic substances

The EEM fluorescence spectra of unheated soil humic substances (UHS) and heated soil humic substances (HHS) in the absence and presence of Hg(II) were shown in Fig. 1. Peak I at Ex/Em = 365–370/470–474 nm observed in the EEM spectra was assigned to UV humic-like fluorescence and peak II at Ex/Em = 270–275/468–472 nm was visible humic-like fluorescence (Coble 1996). Peaks I

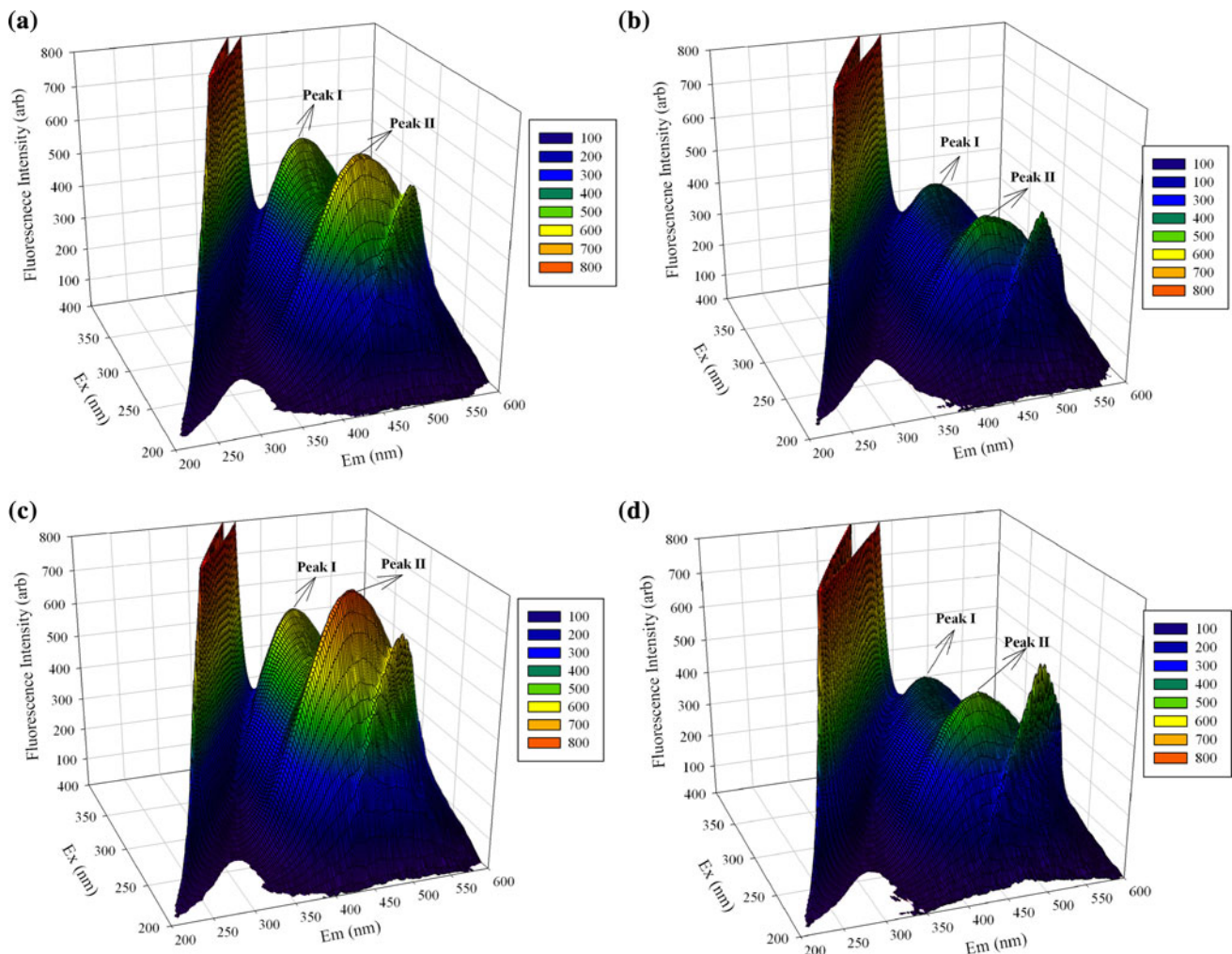


Fig. 1 Three-dimensional EEM plots of humic substances solutions: **a** UHS unheated soil humic substances, **b** UHS + 133 μM Hg(II), **c** HHS heated soil humic substances, **d** HHS + 133 μM Hg(II)

and II represented fluorophores I and II in UHS and HHS, respectively. Peaks I and II were considered to be related to the presence of unsaturated functional groups like carbonyl and carboxylic groups in humic substances (Fu et al. 2007). Both fluorophores I and II could be further identified as microbial humic substances (Yamashita and Jaffe 2008).

Both ionic strength and pH can influence the fluorescence intensity of fluorophores (Merdy et al. 2009; Zhang et al. 2010). In this study, humic substances solutions were prepared from the same concentration of KOH solution (0.1 M), thus the influence of ionic strength and pH was not considered. In comparison with the positions of peaks I and II for UHS, the positions for HHS showed blue shifts (peak I shifted from Ex/Em = 370/474 nm to Ex/Em = 365.5/470 nm, and peak II shifted from Ex/Em = 275/474 nm to Ex/Em = 270/468 nm). These slight blue shifts indicated that heat treatment might have effect on fluorescent properties of humic substances. In addition, the fluorescence intensities of peaks I and II for HHS were greater than those for UHS. This suggested that the activity of fluorophores in humic substances was enhanced after heat treatment. Peak I for the UHS (Table 1) split into two peaks after titrated by Hg(II). This might result from the changes in conformation or rigidity (Wu et al. 2004) and the alteration of electronic structure of humic substances during the binding process (Zhao and Nelson 2005).

It can be seen from Fig. 2 that the fluorescence intensities of peaks I and II for two humic substances decreased markedly with the addition of Hg(II), indicating that humic-like fluorophores in the humic substances solutions reacted strongly with Hg(II). When metal ions are bound to humic substances, they may alter the fluorescence feature of humic substances (Zhao and Nelson 2005). Once the concentration of Hg(II) exceeded 1.17×10^{-4} M, the changes in fluorescence intensities became slight and tended to level off thereafter.

Table 1 The Ex/Em values and intensities of UHS and HHS in absence and presence of 133 μ M Hg(II)

Peak	In absence of Hg(II)		In presence of 133 μ M Hg(II)	
	Position (Ex/Em)	Intensity	Position (Ex/Em)	Intensity
UHS peak I	370.0/474.0	517.7	365.0/470.0 ^a	364.8
			370.0/474.0 ^a	364.0
UHS peak II	275.0/474.0	613.1	275.0/472.0	426.1
HHS peak I	365.5/470.0	560.1	270.0/468.0	361.1
HHS peak II	270.0/468.0	747.7	275.0/470.0	468.9

UHS Unheated humic substances, HHS heated humic substances

^a Peak I of UHS split into two peaks

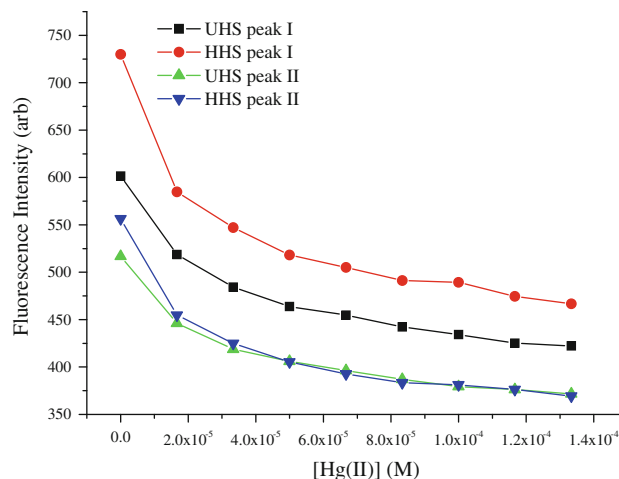


Fig. 2 Variation of fluorescence intensities of peaks in EEM spectra of humic substances with increasing Hg(II) concentration up to 133 μ M. [Hg(II)] is the Hg(II) concentration. (UHS unheated soil humic substances, HHS heated soil humic substances)

Fluorescence titration

Fluorescence quenching data were analyzed with the Stern–Volmer equation (Gauthier et al. 1986)

$$\frac{F_0}{F} = 1 + k_q \tau_0 [\text{Hg(II)}] = 1 + K_{SV} [\text{Hg(II)}] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher [Hg(II) in this study], respectively. Parameter k_q is an energy transfer rate ($\text{L mol}^{-1} \text{s}^{-1}$), while τ_0 refers as to lifetime of fluorescence(s), and [Hg(II)] is the concentration of Hg(II). The Stern–Volmer constant, K_{SV} , is the slope of the plot of F_0/F versus the concentration of quencher.

Poor linear relationship ($R^2 = 0.81\text{--}0.87$) between F_0/F and [Hg(II)] was observed for all peaks (Fig. 3). It has been reported that a concave-down response is usually reflective of the existence of two fluorophore populations at an individual peak (Lakowicz 1999; Zhao and Nelson 2005). One population is accessible to the quencher, and the other is inaccessible to the quencher. In the present study, the modified Stern–Volmer equation or Lehrer equation was therefore further used to depict the fluorescence quenching (Lehrer 1971; Lu and Jaffe 2001; Zhang et al. 2010). In the modified equation, both the quenchable fluorophores and the quencher inaccessible fluorophores are considered. According to (Lakowicz 1999), the total fluorescence (F_0) fluorophores in the absence of quencher, F_0 equals to F_{0a} and F_{0b} .

$$F_0 = F_{0a} + F_{0b} \quad (2)$$

Where F_{0a} is the fluorescence intensity of the fluorophore moieties which can complex with quencher and F_{0b} is the

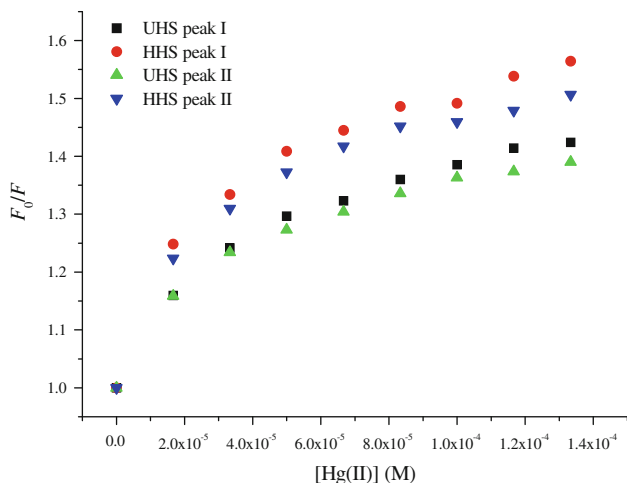


Fig. 3 Stern–Volmer plots of F_0/F versus $[Hg(II)]$. (UHS unheated soil humic substances, HHS heated soil humic substances)

fluorescence intensity of the inaccessible fluorophore moieties. The fluorescence intensity of the accessible fraction is reduced in the presence of quencher while there is no change in the fluorescence intensity of the inaccessible fraction. The observed intensity is given by

$$F = \frac{F_{0a}}{1 + K_a[Hg(II)]} + F_{0b} \tag{3}$$

where K_a is the Stern–Volmer quenching constant of the accessible fraction. From Eqs. (2) and (3), the modified Stern–Volmer equation can be obtained:

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a K_a [Hg(II)]} + \frac{1}{f_a} \tag{4}$$

$$f_a = \frac{F_{0a}}{F_{0a} + F_{0b}} \tag{5}$$

where F_0 and F are the fluorescence intensities in the absence and presence of $Hg(II)$, respectively; f_a is the fraction of the accessible fluorophore; K_a is the effective quenching constant or conditional stability constant; and $[Hg(II)]$ is the concentration of $Hg(II)$. A plot of $F_0/(F_0 - F)$ versus $1/[Hg(II)]$ yields the slope ($1/f_a K_a$) and intercept ($1/f_a$).

The modified Stern–Volmer plots for titration of humic substances with $Hg(II)$ were shown in Fig. 4. The parameters, f_a and $\log K_a$, of fluorophores at peaks I and II were calculated and summarized in Table 2. In the present study, the titration data were well represented by the modified Stern–Volmer equation ($R^2 > 0.99$). In other studies, the fluorescence quenching data of humic substances in water by $Hg(II)$ were also well described by the modified Stern–Volmer equation (Esteves da Silva et al. 1998; Lu and Jaffe 2001). The values of $\log K_a$ of humic substances– $Hg(II)$

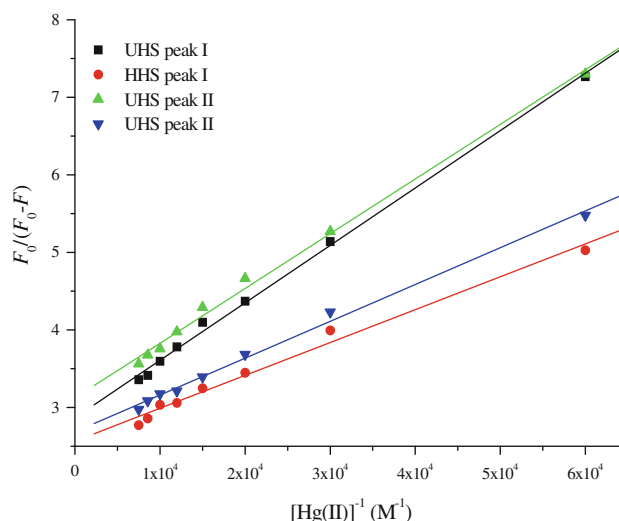


Fig. 4 The modified Stern–Volmer plots for the fluorescence quenching data of humic substances by $Hg(II)$ at $25 \pm 0.1^\circ C$. (UHS unheated soil humic substances, HHS heated soil humic substances)

complexes ranged from 4.59 ± 0.04 to 4.78 ± 0.05 , which were close to those reported in earlier studies using other methods (Yin et al. 1997; Bai et al. 2008). Yin et al. (1997) determined the value of $\log K_a$ of soil humic substances– $Hg(II)$ complexes to be 4.7 using an iodide selective electrode. Bai et al. (2008) measured the values of $\log K_a$ for complexation of Amherst humic acids with $Hg(II)$ to be 4.85 using UV absorbance titration and 4.94 using fluorescence quenching titration. In addition, the values of $\log K_a$ and f_a of humic substances– $Hg(II)$ complexes were also comparable with those of dissolved organic matters (DOM)– Hg complexes (Lu and Jaffe 2001; Wu et al. 2004; Fu et al. 2007). Fu et al. (2007) reported the values of $\log K_a$ of Hg complexation of humic-like substances in DOM were 5.62 and 5.01.

The values of $\log K_a$ for peaks I and II of HHS– $Hg(II)$ complexes were 4.78 ± 0.05 and 4.75 ± 0.04 , respectively, which were larger than those of UHS– $Hg(II)$ complexes. This suggested that humic substances in heated soil can be more easily quenched by $Hg(II)$ and could form more stable complexes with $Hg(II)$ than humic substances in unheated soil. The values of f_a decreased from 38.97 ± 2.04 and $37.21 \pm 1.44\%$ to 34.86 ± 1.57 and $32.01 \pm 1.93\%$ for peaks I and II of HHS, respectively, indicating more fluorophores in HHS were accessible to $Hg(II)$.

Binding constants and binding sites

When small molecules bind independently to a set of equivalent sites on a macromolecule, the binding constant (K_b) and the number of binding sites (n) would be derived from the modified Hill Equation (6) (Hill 1985):

Table 2 Fluorescence quenching and binding parameters for humic substances–Hg(II) interactions

Samples	Peak	f_a (%)	$\log K_a$	SD ^a	R^2 ^b	$\log K_b$	n	SD ^c	R^2 ^d
UHS	I	34.86 ± 1.57	4.59 ± 0.04	0.07	0.997	1.45 ± 0.09	0.47 ± 0.02	0.02	0.987
	II	32.01 ± 1.93	4.65 ± 0.05	0.09	0.996	1.26 ± 0.08	0.42 ± 0.02	0.02	0.988
HHS	I	38.97 ± 2.04	4.78 ± 0.05	0.09	0.995	1.26 ± 0.07	0.39 ± 0.02	0.01	0.990
	II	37.21 ± 1.44	4.75 ± 0.04	0.07	0.999	1.22 ± 0.01	0.39 ± 0.02	0.02	0.982

UHS Unheated humic substances; HHS heated humic substances

$P < 0.0001$

^a Standard deviation for Eq. (4)

^b Linear correlation coefficient for Eq. (4)

^c Standard deviation for Eq. (6)

^d Linear correlation coefficient for Eq. (6)

$$\log \frac{F_0 - F}{F} = \log K_b + n \log[\text{Hg(II)}] \quad (6)$$

where F_0 and F of the fluorophore are the fluorescence intensities in the absence and presence of Hg(II), respectively; $F_0 - F$ is the fluorescence intensities of the fraction of quenched fluorophore, indicating the fraction of ligand binding sites; K_b is the binding constant; n is the number of binding sites.

As shown in Fig. 5 and Table 2, the plots of $\log[(F_0 - F)/F]$ versus $\log[\text{Hg(II)}]$ exhibited good linearity ($R^2 > 0.98$). The values of $\log K_b$ and n for all humic substances were listed in Table 2. For both UHS and HHS, the values of K_b and n of peak II were larger than those of peak I. It was consistent with the fluorescence spectroscopy results that the fluorescence intensities of peak I were weaker than peak II. In this study, the values of n for peaks I and II of UHS and HHS were less than 1, suggesting that more than one Hg(II) binding sites in humic substances

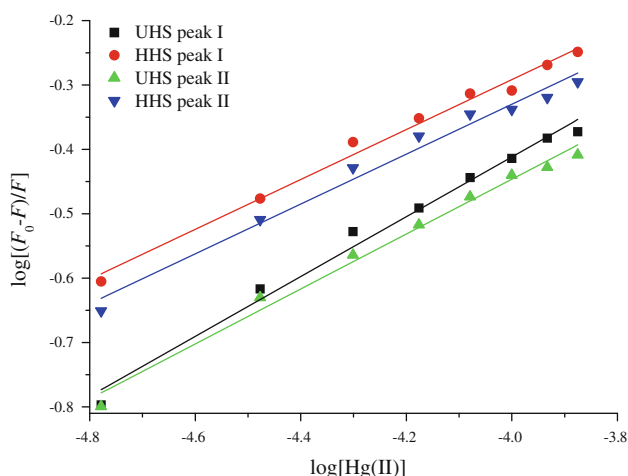


Fig. 5 Plots of $\log(F_0 - F)/F$ versus $\log[\text{Hg(II)}]$ for binding of humic substances to Hg(II) at $25 \pm 0.1^\circ\text{C}$. (UHS unheated soil humic substances; HHS heated soil humic substances)

negatively competed to form humic substances–Hg(II) complexes and more binding sites in humic substances were required for binding to Hg(II).

The values of $\log K_b$ for peaks I and II of UHS were greater than those for peaks I and II of HHS, implying that heat treatment reduced the binding capacity of Hg(II) of each binding site in humic substances. In addition, n was larger for peaks I and II in HHS than UHS. This implies that the heat treatment results in more binding sites in humic substances to bind Hg(II). This point was in line with the changes in the values of f_a which had been discussed in the above section.

Conclusions

This study has expanded knowledge of effects of high temperature on fluorescence features of humic substances derived from sandy soil and their binding to Hg(II). Compared with UHS, slight wavelength shifts were observed in the positions of peaks I and II in the EEM spectra of HHS, implying changes in the fluorescence properties of humic substances. Heat treatment reduced the Hg(II) binding capacity of each binding site of humic substances, increased the binding sites in humic substances that can interact with Hg(II), and formed more stable humic substances–Hg(II) complexes. The findings of the present study suggest that transport and fate of Hg(II) in arid land might be affected significantly due to the modification of humic substances caused by high soil temperature in summer.

Other methods including infrared spectroscopy (IR) and nuclear energy resonance (NMR) should be employed complementarily to better understand the mechanisms involved in the heat treatment process. Additionally, due to the complex nature of soil, others factors, including microbial activity, should be considered to further

understand the fate, chemical forms, mobility, bioavailability and ecotoxicity of Hg in high temperature environment.

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